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Note

Application of isotachophoresis in enzymology

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The theoretical aspects of isotachophoresis have been considered by several workers[1,2]. In enzyme assay, isotachophoresis has been used only a few times to separate the ionic components before the addition of the enzyme and after complete conversion[2–5]. In most of these reactions, NADPH/NADP or ATP/ADP was involved[*].

Another class of enzymatic reactions consists of those reactions in which NADH/NAD is involved. An arbitrary example of this class is

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{LDH}}{\rightleftharpoons} \text{NAD} + \text{Lactate}$$
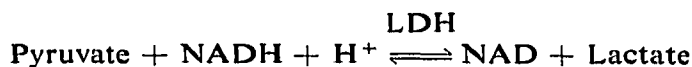
Pyruvate, lactate and NADH can easily be separated by isotachophoresis but with NAD difficulties arise because of the very low effective mobility. If we succeed in finding a terminating ion with a lower effective mobility than that of NAD, then we could separate the components not only of the above reaction but also of the whole class of oxidoreductase reactions in which NADH/NAD is involved.

OPERATIONAL SYSTEM AND PURIFICATION OF THE TERMINATOR

The separation of the above reaction components can be achieved satisfactorily at a pH of about 4. The difficulty is to find a terminating ion with a sufficiently low effective] mobility, because there is a greater chance that impurities in the terminating electrolyte with a greater effective mobility will interfere in the isotachophoretic analysis. The impurities migrate as a moving boundary system through the sample zones and therefore purification of the terminator is necessary.

Caprylic acid was found to have a sufficiently low mobility and could be purified by isotachophoresis. First, caprylic acid is extracted a few times with water and then the terminating electrolyte compartment is filled with the extracted caprylic acid and water. A ring-shaped electrode is placed in the upper caprylic phase so that after

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[*] Abbreviations used: ADP = Adenosine-5′-diphosphate; ATP = adenosine-5′-triphosphate; LDH = lactate dehydrogenase; MES = morpholinoethanesulphonic acid; NAD = nicotinamide-adenine dinucleotide (oxidized); NADH = nicotinamide-adenine dinucleotide (reduced); NADPH = nicotinamide-adenine dinucleotide phosphate (reduced).

a potential has been applied over the compartments containing the terminator and leading electrolytes, the potential remains over the entire aqueous phase. Those impurities in the aqueous phase which have a greater effective mobility than caprylate then migrate out of the terminator phase. This can easily be detected with a conductimetric and/or a UV detector. By replenishing the aqueous phase several times, the terminator thus becomes purer. This purification method can be used not only for caprylic acid but for all terminators that are slightly soluble in water but to a sufficient extent for isotachophoresis. The application of a counter-flow technique accelerates the procedure.

However, caprylic acid is disappointing as a terminator in practice. It adheres to the capillary tube and to the micro-sensing electrodes of the conductivity detector, and forms adsorption layers that influence the step heights of the sample components and the terminator. Because of this, the capillary must be rinsed thoroughly after each analysis.

Another useful terminator is MES. Purification is still necessary, for although a chemical reagent may be of *pro analysi* (p.a.) grade, this does not mean that it is suitable as a terminator in isotachophoretic analysis. MES is purified by precipitation with ethanol from a saturated solution in double-distilled water. After filtration, the crystals are washed with acetone.

Fig. 1 shows an example of the various stages in the purification. A shows the conductimetric electropherogram of p.a. MES as terminator, B shows the same MES after two recrystallizations and C shows the terminator after complete purification. It can be seen that the step-height increases during the purification, as expected.

In Table I the operational system with which the analyses of the enzymatic reaction mixture were performed is given.
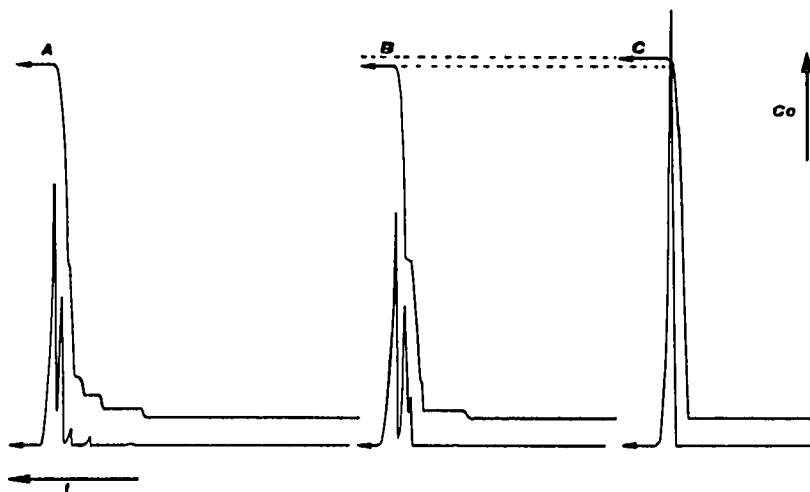

Fig. 1. Some stages in the purification of MES. A, electropherogram of *pro analysi* MES; B, as A, after two recrystallizations; C, as A, after a complete purification. Special attention should be paid to the shift in step-height of the terminating ion (qualitative information after purification). The current was stabilized at 70 μA; time of analysis, 12 min; recorder chart speed, 6 cm/min; t = time; Co = conductivity.

TABLE I

OPERATIONAL SYSTEM FOR ISOTACHOPHORETIC ANALYSIS AT pH 4.75 (ANIONS)

Electrolyte	Property	Condition
General	Solvent	Water
	Stabilizing agent	None
	Electric current	70–100 μA
	Temperature	22°
Leading electrolyte	Conc. anion	0.01 M HCl (p.a. grade)
	Conc. buffer	ε-Aminocaproic acid, adjusted to pH 4.5, histidine to pH 4.75
	Additive	0.05 % polyvinyl alcohol
Intermediate electrolyte	Ionic strength	Adjusted approximately to the ionic strength of the leading electrolyte
	Additive	None
Terminating electrolyte	Conc. anion	$ca.$ 0.01 N MES
	pH	$\ll$4.75
	Additive	None

TERMINATION OF AN ENZYMATIC REACTION

A method of following an enzymatic reaction is the sampling method, in which the reaction is terminated after a suitable time interval. After the isotachophoretic separation, the concentrations of all of the ionic components of the reaction mixture can be measured from the electropherogram.

We examined and compared several techniques for terminating the enzymatic reaction.

First, we decreased the activity of the enzyme by decreasing the pH. This technique proved not to be useful because the introduction of hydrochloric acid did not decrease the reaction rate sufficiently and the electrophoretic analysis was affected by overloading the capillary.

A better technique was to terminate the enzymatic reaction by inhibition of the enzyme. A disadvantage was that an extra component (the inhibitor) had to be separated. The effective mobility of the inhibitor must differ sufficiently from the mobilities of the sample ions.

Attempts to terminate the reaction by filtration of the enzyme over a protein filter failed. The pores were so small that filtration took too long.

The best results were obtained by thermal denaturation of the enzyme in a high-frequency field. For this purpose, a melting-point tube is filled (using a syringe) with the reaction mixture. This tube is wrapped with a Kanthal spring and placed in the high-frequency field for $ca.$ 8 sec. The temperature of the spring is the Curie temperature of Kanthal and the temperature in the tube is $ca.$ 80°.

EXPERIMENTAL AND RESULTS

The analyses were performed in the apparatus described earlier[5]. All chemicals used were of the highest commercial grade available and obtained from Merck (Darmstadt, G.F.R.), except for pyruvate, NADH and the enzyme LDH, which were
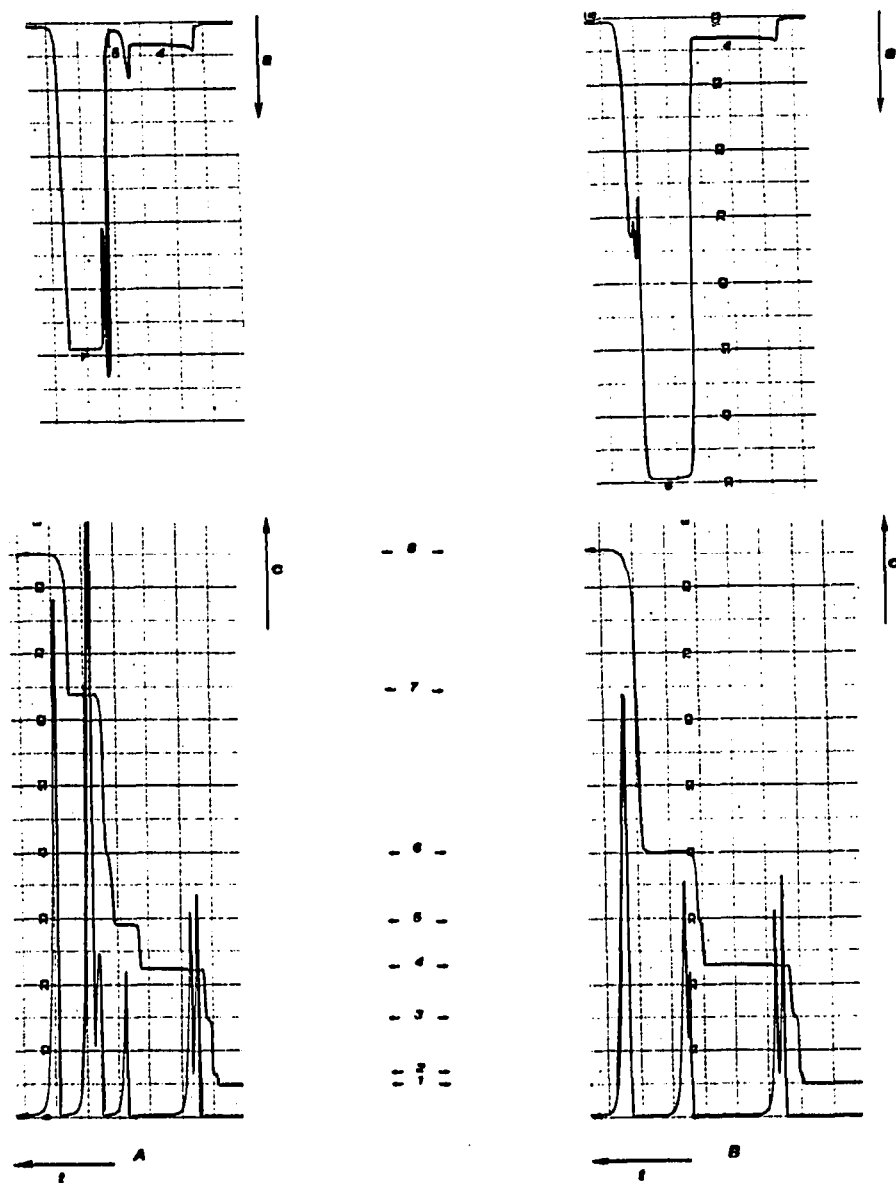
Fig. 2. Enzymatic conversion of pyruvic acid into lactic acid by LDH (pig heart) shown by iso-tachophoresis. A, reaction mixture after conversion; B, reaction mixture before conversion. Time of analysis, 12 min; recorder chart speed, 6 cm/min; current, stabilized at 70 μA; t = time; c = conductivity; a = UV absorption (254 nm). 1 = Chloride; 2 = sulphate; 3 = unidentified; 4 = pyruvate; 5 = lactate; 6 = NADH; 7 = NAD; 8 = MES (terminator).

obtained from Boehringer (Mannheim, G.F.R.). Double-distilled water was used as the solvent.

When separating weak acids, large pH shifts can occur[2]. For this reason, the leading electrolyte is first buffered to pH 4.5 with ε-aminocaproic acid (pK value 4.37)
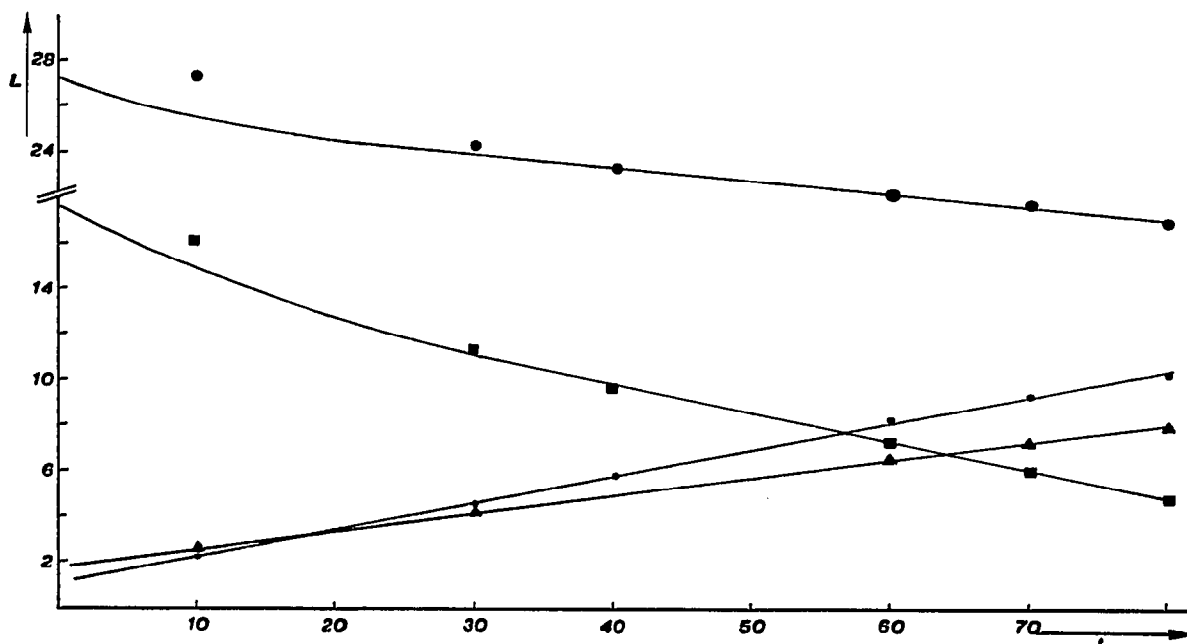
Fig. 3. Zone lengths of the isotachophoretic zones as a function of the reaction time. ●, Pyruvate; ■, NADH; ●, NAD; ▲, lactate. L = Step-height measured isotachophoretically; t = reaction time.

and then to pH 4.75 with histidine (pK value 6.02). Histidine will buffer the zones in the rear.

For the experiment, NADH and pyruvate were dissolved in water to concentrations of 0.011 and 0.040 M, respectively, and buffered with Tris to pH 7.6. The reaction mixture was controlled thermostatically at 5° and the reaction was started by adding 2.5 μl of LDH suspension. Every 10 min, a sample of this mixture was removed and 0.3 μl analyzed by isotachophoresis. The zones were detected with a UV and a conductimetric detector. Fig. 2B shows the electropherogram of the reaction mixture before the reaction is started, and Fig. 2A shows the electropherogram after the reaction is completed.

Fig. 3 shows the zone lengths of the reaction components as found in the electropherograms as a function of the reaction time. NAD and lactate show a linear relation-

TABLE II

BALANCE OF REACTION COMPONENTS AFTER DIFFERENT REACTION TIMES

Reaction time (min)	Pyruvate (nmole)	Lactate (nmole)	NADH (nmole)	NAD (nmole)
40	−148.4	+134.8	−127.2	+128.2
60	−195.5	+196.4	−180.6	+183.4
70	−206.3	+227.2	−197.8	+205.5
80	−235.3	+250.3	−218.2	+225.4

ship, while during the first 30 min NADH and pyruvate show a non-linear relationship.

For several reaction times, we made a balance for all of the reaction components (Table II).

For 40, 60 and 70 min, the amounts of pyruvate consumed agree reasonably well with the amounts of lactate produced, and the same applies for NADH and NAD. The difference between pyruvate/lactate and NADH/NAD is probably caused by errors in the calculation due to the use of impure chemicals and is being studied further.

ACKNOWLEDGEMENT

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